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Description

Technical field of invention

This invention relates to a process for producing specific proteins in bacteria and having them excreted from the bacterial cell and pertains more specifically to inserting the DNA representing the desired non-bacterial protein or part of a protein by recombinant techniques into the plasmid or phage gene for either a periplasmic or an extracellular protein, hereinafter called a "carried protein", transforming a bacterial host with the recombined gene, and culturing the transformed host to excrete the protein. The protein thus produced can be collected by conventional procedures from the culture medium or from the periplasmic space depending upon the choice of carrier protein gene.

Background art

It is known to insert DNA representing a specific protein into the gene for an intracellular protein. Itakura et al, *Science* 198, 1056 (1977). The problem however is that proteins made in this way are mixed with other intracellular proteins and are therefore subject to degradation by enzymes within the cell so that there is a problem in obtaining the desired protein product in purified form.

Disclosure of invention

The foregoing problem is avoided in accordance with our invention by providing a method of making a selected protein or portion thereof by inserting DNA representing the selected protein or portion thereof into a bacterial gene, characterized by cleaving the bacterial gene for an extracellular or periplasmic carrier protein, inserting into the cleavage site by a recombinant step a non-bacterial DNA fragment which codes for the selected protein or portion thereof, transforming a bacterial host with the recombined gene, and culturing the transformed bacteria to excrete the selected protein or portion thereof.

By way of example, by employing a gene for a carrier protein which has a leader sequence of hydrophobic amino acids at its amino terminus and which is normally excreted through the membrane of the cell within which it is made, with cleavage of the hydrophobic leader sequence during excretion, a selected protein can be produced which can be recovered either from the periplasmic space or from the medium in which the bacterium is grown, depending upon the choice of carrier protein. In this way contamination from the other proteins within the bacterium is avoided while achieving greater stability by avoiding the enzymes within the bacterial cell which degrade foreign proteins.

Among the bacterial genes for carrier proteins which genes can therefore be employed in the present invention are the genes for antibiotic resistance, such as the gene for penicillin resistance or possibly penicillinase, the gene for chloramphenicol resistance, or the gene for tetracycline resistance, as well as the gene for alkaline phosphatase and the gene for bacterial ribonuclease.

Genes or DNA fragments which code for a variety of proteins or portions thereof can be inserted in the bacterial carrier protein gene by the process of the present invention. These proteins include a variety of non-bacterial proteins such as eukaryotic cell proteins and viral proteins. Of particular importance are eukaryotic cell proteins such as insulin, human growth hormone, interferon and other pharmacologically active proteins. These are synthesized by their respective genes as pre-proteins or precursor proteins having at their amino terminus a series of hydrophobic amino acids. This hydrophobic leader sequence is not identical to that for the bacterial proteins which are excreted through the bacterial membrane. Therefore, the fact that pre-insulin or other pre-proteins of higher cells contain a hydrophobic leader sequence is in itself no basis for expecting that such a pre-protein could be matured in the bacterial cell even if it could be synthesized within the cell. Moreover, the process of the present invention, in addition to providing for the synthesis within and excretion from bacterial cells of matured proteins of eukaryotic cells, which are of known utility, also makes possible the synthesis in and excretion from bacterial cells of other extracellular products of commercial interest. These include other fused proteins and fused proteins consisting -of carrier proteins, as defined above, which carry specific determinants, for example, viral antigens such as coat proteins or other antigenic proteins of viruses. These latter fused proteins are useful in the manufacture of vaccines, being capable because of their antigenic character of inducing generation of an immune response specific to the viruses. Such vaccines will be unusually safe because they will not contain any live or inactivated virus material. Furthermore, it is possible by this process to construct vaccines for viruses which cannot be grown in culture.

Brief description of the drawings

Figs. 1, 2 and 3 of the drawing show the complete base sequence for the E. *coli* penicillinase gene carried on the plasmid pBR322 along with the corresponding amino acid sequence of the protein for which it codes.

Best mode for carrying out the invention

The following specific example is intended to illustrate more fully the nature of the present invention

without acting as a limitation upon its scope.

Example

There was employed as the carrier protein *E. coli* [*Escherichia coli*] penicillinase, the gene for which is carried on the small plasmid pBR322. A restriction enzyme map of this gene is shown in the drawing. This plasmid vector has been described by Bolivar *et al.*, Gene, 2, 95-113 (1977). As the host bacteria we employed *E. coli* $_x$1776*; 1 see Curtiss *et al.* in Recombinant Molecules: Impact on Science and Society. Proceedings of the Tenth Miles International Symposium, eds. Beers & Bassett, 45-56 (1977). The host-vector combination is a certified EK2 system, certified by the United States National Institute of Health (NIH), July 7, 1977.

The plasmid carries a Pst [providencia stuartii endonuclease] restriction site of the penicillinase gene corresponding to the position of amino acids 181 and 182, as shown in the drawing. Double stranded cDNA was synthesized from RNA containing preproinsulin mRNA (PPI-mRNA) isolated from an X-ray induced, transplantable rat B-cell tumor (Chick *et al.*, Proc. Natl. Acad. Sci. USA [P.N.A.S.], *74*, 628-632 [1977]). Batches of 20 g each of frozen tumor slices were ground with sterile sand with mortar and pestle and the cytoplasmic RNA purified from a post-nuclear supernatant by Mg^{2+} precipitation (Palmiter, Biochemistry, *13*, 3603-3615 (1974)) followed by extraction with phenol and chloroform. This RNA was further purified by oligo-dT-cellulose chromatography (Aviv et al, P.N.A.S., *69*, 1408-1412 (1972)) and used directly as template for double-stranded cDNA synthesis, as described (Efstratiadis *et al.*, Cell 7, 279-288 (1976)), except that a specific $p(dT)_8$ dG-dC primer (Collaborative Research) was utilized for reverse transcription. The concentrations of RNA and primer were 7 mg/µl and 1 mg/µl, respectively. All four α-^{32}P-dNTPs were at 1.25 mM (final specific activity 0.85 Ci/m mole). The reverse transcript was 2% of the input RNA, and 25% of it was finally recovered in the double-stranded DNA product.

The double-stranded cDNA was inserted into the Pst site of plasmid pBR322 by the following procedure: pBR322 DNA (5.0 µg) was linearized with Pst and approximately 15 dG residues were added per 3′ end by terminal transferase at 15°C in the presence of 1 mM Co^{2+} (Roychoudhury *et al.*, Nucleic Acids Res., *3*, 101-116 (1976)) and 100 µg/ml autoclaved gelatin. The same procedure was used to add dC residues to 2.0 µg of double-stranded cDNA. The reaction mixtures were extracted with phenol, and ethanol precipitated. The dC tailed double-stranded cDNA was electrophoresed in a 6% polyacrylamide gel under native conditions. Following autoradiography, molecules in the size range of 300 to 600 base pairs (.5 µg) were eluted from the gel (Efstratiadis *et al.* in Methods in Molecular Biology, *8*, 1-124 (1976)). The eluted double-stranded cDNA was concentrated by ethanol precipitation, redissolved in 10 mM Tris pH 8, mixed with 5 µg dG tailed pBR322 and dialized versus 0.1 M NaCl, 10 mM EDTA, 10 mM Tris pH 8. The mixture (4 ml) was then heated at 56° for 2 minutes, and annealing was performed at 42° for 2 hours. The hybrid DNA was used to transform *E. coli* 1776. The use of oligo dCdG joins regenerates the Pst cuts so that the insert may be later excised.

Transformation of *E. coli* $_x$1776 (an EK-2 host with pBR322, an EK-2 vector) was performed in a biological safety cabinet in a P3 physical containment facility in compliance with N.I.H. guidelines for recombinant DNA research published in the *Federal Register*, July 7, 1976.

$_x$1776 was transformed by transfection procedure (Enea *et al.*, J. Mol. Biol. *96*, 495-509 (1975)) slightly modified as follows: $_x$1776 was grown in L Broth [10 gms tryptone, 5 gm yeast extract, 5 gm NaCl (Difco)] supplemented with 10 µg/ml diamino-pimelic acid and 40 µg/ml thymidine (Sigma) to A_{590} of 0.5. A 200 ml portion of cells were sedimented at 3000 rpm and resuspended by swirling in 1/10 volume of cold buffer containing 70 mM $MnCl_2$, 40 mM NaAc pH 5.6, 30 mM $CaCl_2$ and kept on ice for 20 minutes. The cells were repelleted and resuspended in 1/30 of the original volume in the same buffer. The annealed DNA (2 ml) was added to the cells. Aliquots of this mixture (0.3 ml) were placed in sterile tubes and incubated on ice 60 minutes. The cells were then placed at 37° for 2 minutes. Broth was added to each tube (.7 ml) and the tubes incubated at 37° for 15 minutes. A 200 µl portion of the cells was spread on sterile nitrocellulose filters (Millipore) overlaying agar plates containing 15 µg/ml tetracycline. (The filters were boiled to remove detergents before use). The plates were incubated at 37° for 48 hours. Replicas of the filters were made. The nitrocellulose filters containing the transformants were removed from the agar and placed on a layer of sterile Whatman filter paper. A new sterile filter was placed on top of the filter containing the colonies and pressure was applied with a sterile velvet cloth and a duplicate block. A sterile needle was used to key the filters. The second filter was placed on a new agar plate and incubated at 37° for 48 hr. The colonies on the first filter were screened by the Grunstein-Hogness (P.N.A.S., 72, 3961-3965 (1975)) technique,

1 *A deposit of the Escherichia coli X 1776 has been placed with and made available to the public at the American Type Culture Collection, Rockville, Maryland, U.S.A. and has been assigned ATCC No. 31244.

using as probe an 80-nucleotide long fragment produced by Hae III digestion of high specific activity cDNA copied from the rat oligo-dT bound RNA. Positive colonies were rescreened by the HART method (Paterson *et al.*, P.N.A.S., *74*, 4370-4374 (1977)) as follows: Plasmid DNA (about 3 μg) was digested with Pst, ethanol precipitated and dissolved directly into 20 μl dionized formamide. After heating for one minute at 95° each sample was placed on ice. After the addition of 1.5 μg oligo (dT)-cellulose bound RNA, PIPES at pH 6.4 to 10 mM and NaCl to 0.4 M, the mixtures were incubated for 2 hr at 50°. They were then diluted by the addition of 75 μl H_2O and ethanol precipitated in the presence of 10 μg wheatgerm tRNA, washed with 70% ethanol, dissolved in H_2O and added to a wheatgerm cell-free translation mixture (Roberts *et al.*, P.N.A.S., *74*, 2330-2334 (1973)). After three hours at 23°C, duplicate 2 μl aliquots were removed for trichloroacetic acid precipitation; the remainder of the reaction mixture was treated with ribonuclease, diluted with immunoassay buffer, and analyzed for the syntheses of immunoreactive preproinsulin by means of a double antibody immunoprecipitation (Lomedico *et al.* Nucleic Acids Res., *3*, 381-391 (1976)). The washed immunoprecipitates were dissolved in 1 ml of NCS (Amersham) and counted in 10 μl of Omnifluor (New England Nuclear) by liquid scintillation.

One colony was identified by the HART Screening. The Pst excisable insert was sequenced by the method of Maxam and Gilbert (P.N.A.S., *74*, 560-564 (1977)) to show that it corresponded to the sequence of rat preproinsulin I. This insert, labeled by nick translation with DNA polymerase I was used to screen 200 transformants with the Grunstein-Hogness assay. There were identified 48 clones hybridizing to the rat preproinsulin cDNA probe.

These 48 clones of transformed *E. coli* $_x$1776 were screen using an *in situ* radioimmuno-assay technique to determine whether the clones were producing insulin antigens and whether they were producing fused polypeptide chains, one end of which being insulin antigen and the other end penicillinase (the bacterial carrier protein) antigen. Presence of the fused polypeptide chains would indicate that the clones contained genes which were the products of the fusion of the bacterial gene for penicillinase with the eukaryotic cell gene for insulin. Such fused polypeptide chains were in fact found, using the technique to be described below. The technique takes advantage of the fact that the fused proteins being searched for contain two antigenic ends, each of which will bind to its respective specific antibody. A specific antibody was laid down on a plastic disk, the antigenic protein from lysed bacterial cells placed in contact with this disk, then the disk was rinsed and exposed to radioactive antibodies. A protein molecule will bind to the antibody fixed to the plastic with one antigenic determinants and will bind in turn a radioactive antibody with a second determinant. If anti-penicillinase is on the disk and anti-insulin is labelled, after the "sandwich" is washed, the only points of radioactivity remaining will mark the presence of fused proteins. In more detail, the method was as follows:

Each 8.25 cm diameter disk of clear polyvinyl (PV) 8 mm thick (Dora May Co., New York) was flattened between sheets of smooth paper. In a glass petri disk, each disk was then placed upon the surface of a liquid containing 10 ml of 0.2 $MNaHCO_3$, at pH 9.2, containing 60 μg/ml IgG. After 2 minutes or longer at room temperature, the disk was removed and washed twice with 10 ml of cold wash buffer (WB) which consisted of phosphate-buffered saline, 0.5% normal guinea pig serum, 0.1% bovine serum albumin and 0.3 mg/ml streptomycin sulfate. Each disk was used immediately after washing.

Antigens were released from bacterial cells by transferring colonies onto 1.5% agarose containing 0.5 mg lysozyme/ml, 30 mM Tris pH 8, and 10 mM EDTA. The IgG-coated surface of a PV disk was placed face down on the agarose and bacterial colonies and left for 60 minutes at 4°. Each disk was then removed and washed 3 times with 10 ml of cold WB. This step completed the immunoadsorption of antigen onto the solid-phase antibody layer.

Reaction of the ^{125}I-labeled antibodies with the antigen now adhering to the disks was done by setting 1.5 ml WB containing 5 x 10^6 cpm (γ emission) ^{125}I-IgG onto the center of an 8.25 cm diameter flat disk of ordinary nylon mesh which had been placed in the bottom of a petri dish. The mesh served as a spacer. A disk treated as in the earlier steps then was placed facedown on the mesh and solution and incubated overnight at 4°. Each disk was then washed twice with 10 ml cold WB and twice with water, and allowed to dry at room temperature. At this point, fused proteins had bound to both the ordinary and radioactively labeled layers of IgG. These proteins were then detected with conventional autoradiography technique using Kodak No. Screen Film or Kodak X-OMAT R film and a DuPont Cronex Lighting plus intensifying screen as described for example by Laskey *et al.*, FEBS Lett., *82*, 314-316 (1977). Both anti-insulin and anti-penicillinase IgG fractions were required for the procedure above. The anti-insulin antiserum was a commercially available product obtained from guinea pigs. The rabbit anti-penicillinase anti-serum was produced by injection (1 mg pure) penicillinase (in complete Freund's adjuvant (Difco)) into New Zealand white rabbits. (Booster injections were administered in incomplete Freund's adjuvant (Difco)) 2 and 3 weeks after the initial injection, and the rabbits were bled 1 week later.

The IgG fractions were prepared from each immune serum by ammonium sulfate precipitation followed by DEAE-cellulose (Whatman, DE-52) chromatography in 0.025 M potassium phosphate, pH

7.3, 1% glycerol. Fractions containing the bulk of the flow-through material were pooled, and protein was precipitated by adding ammonium sulfate to 40% saturation. The resulting pellet was resuspended in 1/3 the original serum volume of 0.025 M potassium phosphate, pH 7.3, 0.1 M NaCl, 1% glycerol, and dialized against the same buffer. After dialysis, any residual precipitate was removed by centrifugation. IgG fractions were stored in aliquots at -70°.

Each IgG fraction was radioiodinated by the usual method of Hunter *et al.*, Biochem. J., 91, 43-46 (1964). The 25 µl reaction mixture contained 0.5 M potassium phosphate, pH 7.5, 2 mCi carrier-free $Na^{125}I$, 150 µg IgG and 2 µg chloramine T. After 3 minutes at room temperature, 8 µg of sodium metabisulfite in 25 µl PBS was added, followed by 200 µl PBS containing 2% normal guinea pig serum. The ^{125}I-labeled IgG was purified by chromatography on a Sephadex G-50 column equilibrated with PBS containing 2% normal guinea pig serum. The ^{125}I-IgG elution fraction was diluted to 5 ml with PBS containing 10% normal guinea pig serum, filtered through a sterile Millipore VC filter (0.1 µm pore size), divided into aliquots and stored at -70°. The specific activities were 1.5 x 10^7 cpm/µg.

This screening detected one clone of $_{\chi}1776$ that synthesized and secreted a fused protein showing both penicillinase and insulin antigenic determinant. This protein, recovered from the periplasm, mimics insulin in radioimmunoassays. DNA sequencing shows that this protein is a fusion between penicillinase and proinsulin, the two proteins being connected by 6 glycines between amino acid 182 of penicillinase (Alanine) and amino acid 4, glutamine, of proinsulin. Thus a higher cell hormone has been synthesized in bacteria in an antigenically active form.

It will be appreciated that the DNA sequence for the desired eukaryotic cell protein can be inserted into a Hind II cut corresponding to the position between amino acids 101 and 102 of the protein for which this pBR322 plasmid codes, or into the Taq cut at the position corresponding to amino acid 45. In all cases, if the eukaryotic cell DNA is arranged in phase, by the random addition of tails or by other procedures, it will be expressed as a fused part of the carrier protein; and the protein excreted from the cell. Furthermore, the sequence of the penicillinase gene, as it exists in this plasmid, or in others, can be modified either by mutation, or by direct recombinant DNA techniques such as the insertion of DNA fragments at specific points within the gene, in such way as to insert new restriction cuts that are convenient for splicing. For example, the R1 cut on the plasmid pBR322 can be removed by mutation, and an R1 sequence inserted by ligation into the penicillinase gene. Although this might inactivate the gene, it would not interfere with the use of this region of DNA to synthesize a carrier protein.

The segment of the penicillinase gene DNA between the code for amino acid 23 at the end of the hydrophobic leader and the code for amino acid 45 at the Taq cut for example, can be removed by nibbling back the DNA by a mixture of appropriate enzymes. One such mixture is the lambda exonuclease which will chew back the DNA strand from the 5' end, together with the enzyme S1, which will remove the single stranded overhand. Another such mixture is T_4 DNA polymerase which will chew back the 3' end of one DNA strand together with S1, which again will remove the single stranded overhang. By controlled digestion the plasmid DNA molecule can be appropriately shortened to the fragment extending from the R1 cut to the point coding for amino acid 23 or to other points on the hydrophobic leader sequence, and such a fragment can be fused to a similarly generated fragment containing the insulin sequence, chewed back enzymatically to a convenient initial point, presumably again, the point where the mature insulin molecule begins. These two fragments can be fused together, for example,, by butt end ligation by the T_4 DNA ligase, and that fusion inserted into the plasmid. That fusion produces a degenerate species of the carrier protein, for which the carrier gene codes for only the *E. coli* hydrophobic leader sequence and the eukaryotic cell gene provides the rest of the structural information. Although such construction can in principle be done exactly, in practice they will probebly be done on a random basis, involving the splicing of a variety of gene fragments whose end points are in interesting regions, and examining the medium surrounding clones of bacteria transformed by the fused fragments to detect antigenic activity by an RIA such as the one described above, as evidence of protein synthesis.

The procedure of the present invention is not restricted to the use of the *E. coli* penicillinase gene, but is applicable to the gene for any excreted protein carried on a multicopy plasmid or on a phage. It is not restricted to insulin, but can be used to fine the expression of the fused protein of any DNA fragment of a virus or eukaryotic cell that carries a coding region that codes, when translated in phase, for antigenic determinants in the viral or eukaryotic cell protein. Thus if fragments of animal virus DNA are inserted into the Pst or Hind II site of the penicillinase gene, some recipient bacterium will synthesize a fused protein which will be recognizable by using the RIA technique, employing antibodies specific to the viral antigen. This fused protein in turn can be purified and used to stimulate an antibody response in an animal or person, either for the production of antibodies directed at specific sites on the virus protein, or as vaccination against the viral antigen. The fused protein will provide helper determinants in such a vaccination, to aid the immune response, although presumably, aggregated states of the fused protein would have to be used in a vaccine. The specific carrier proteins that would be used might be either the

bacterial proteins themselves or still further fusions between the bacterial proteins and other convenient sequences to provide useful helper determinants in carrier protein.

"Difco", "Millipore", "Sephadex" and "Whatman" are Registered Trade Marks.

Claims

Claims for the following Contracting States: BE, CH, DE, FR, GB, IT, LU, NL, SE

1. A method of making a selected fusion protein or fusion polypeptide, which protein or polypeptide is characterized by a non-bacterial protein or polypeptide fused end to end to a portion of a bacterial extracellular or periplasmic carrier protein, characterized by cleaving the bacterial gene for the extracellular or periplasmic carrier protein within the portion of the bacterial gene coding for the bacterial carrier protein, inserting into the cleavage site by a recombinant step a non-bacterial DNA fragment which codes for a protein or polypeptide that is normally excreted through a membrane of the cell within which it is made in nature, transforming a bacterial host with the recombined gene, and culturing the transformed bacteria to excrete the selected fusion protein or fusion polypeptide through a membrane of the transformed bacteria.

2. The method according to claim 1, characterized in that the carrier protein is E.coli penicillinase.

3. The method according to either of claims 1 or 2, characterized in that the selected non-bacterial protein is proinsulin.

4. The method according to claim 1, characterized in that the carrier protein is E.coli penicillinase and the bacterial gene is cleaved at the PstI restriction site.

5. A recombinant DNA molecule comprising a bacterial gene for an extracellular or periplasmic carrier protein and a non-bacterial gene which codes for a selected protein or polypeptide, which protein or polypeptide is normally excreted through a membrane of the cell within which it is made in nature, said non-bacterial gene having been inserted into said bacterial gene within the portion of the bacterial gene coding for the bacterial carrier protein and joined end to end with a portion thereof.

6. The recombinant DNA molecule of claim 5, wherein said non-bacterial gene has been inserted into said bacterial gene at a restriction endonuclease site therein.

7. The recombinant DNA molecule of claim 6, wherein the restriction endonuclease site is the PstI site.

8. The recombinant DNA molecule of claim 5, wherein the bacterial gene is the gene for E.coli penicillinase

Claims for the following Contracting States: AT

1. A method of making a selected fusion protein or fusion polypeptide, which protein or polypeptide is characterized by a non-bacterial protein or polypeptide fused end to end to a portion of a bacterial extracellular or periplasmic carrier protein, characterized by cleaving the bacterial gene for the extracellular or periplasmic carrier protein within the portion of the bacterial gene coding for the bacterial carrier protein, inserting into the cleavage site by a recombinant step a non-bacterial DNA fragment which codes for a protein or polypeptide that is normally excreted through a membrane of the cell within which it is made in nature, transforming a bacterial host with the recombined gene, and culturing the transformed bacteria to excrete the selected fusion protein or fusion polypeptide through a membrane of the transfomed bacteria.

2. The method according to claim 1, characterized in that the carrier protein is E.coli penicillinase.

3. The method according to either of claims 1 or 2, characterized in that the selected non-bacterial protein is proinsulin.

4. The method according to claim 1, characterized in that the carrier protein is E.coli penicillinase and the bacterial gene is cleaved at the PstI restriction site.

5. The method of claim 1, characterized in that said non-bacterial gene has been inserted into said bacterial gene at a restriction endonuclease site therein.

6. The method of claim 5, characterized in that the bacterial gene is the gene for E.coli penicillinase.

7. The method of claim 5, characterized in that the non-bacterial gene is a gene coding for a selected protein or polypeptide selected from the group consisting of proinsulin, interferon, human growth hormone, eukaryotic cell proteins, viral proteins, and other pharmacologically-active products.

Revendications

Revendications pour les Etats contractants suivant: BE, CH, DE, FR, CB, IT, LU, NL, SE

1. Procédé de production d'une protéine de fusion sélectionnée ou d'une polypeptide de fusion sélectionnée, cette protéine ou cette polypeptide est caractérisée par une protéine ou une polypeptide non bactérienne fusionnée bout à bout à une partie d'une protéine porteuse bactérienne extracellulaire ou périplasmique caractérisé en ce qu'on sépare le gène bactérien de la protéine porteuse extracellulaire ou périplasmique à l'intérieur de la partie du gène bactérien codant la protéine porteuse bactérienne, à insérer au site de la division, par une étape recombinant

un fragment d'ADN non bactérien qui code une protéine ou une polypeptide normalement éliminée à travers une membrane de la cellule dans laquelle elle est produite au naturel, à transformer un porteur bactérien par le gène recombiné, et à cultiver la bactérie transformée pour éliminer la protéine de fusion sélectionnée ou la polypeptide de fusion sélectionnée, à travers une membrane de la bactérie transformée.

2. Procédé selon la revendication 1, caractérisé en ce que la protéine porteuse est de la pénicillinase E. Coli.

3. Procédé selon l'une quelconque des revendication 1 et 2, caractérisé en ce que la protéine non bactérienne sélectionnée est de la proinsuline.

4. Procédé selon la revendication 1, caractérisé en ce que la protéine porteuse est de la pénicillinase E. Coli, et en ce que le gène bactérien est séparé au point de Pst de restriction.

5. Molécule d'ADN recombinant comprenant un gène bactérien de protéine porteuse extracellulaire ou périplasmique et un gène non bactérien codant une protéine ou une polypeptide sélectionnée, cette protéine ou cette polypeptide étant normalement éliminée à travers une membrane de la cellule dans laquelle elle est produite au naturel, et ce gène non bactérien ayant été inséré dans le gène bactérien à l'intérieur de la partie de ce gène bactérien codant la protéine porteuse bactérienne, et joint bout à bout à une partie de celui-ci.

6. Molécule d'ADN recombinant selon la revendication 5, caractérisée en ce que le gène non bactérien a été inséré dans le gène bactérien en site d'endonucléase de restriction de celui-ci.

7. Molécule d'ADN de recombinaison selon la revendication 6, caractérisée en ce que le site d'endocucléase de restriction est le site Pst I.

8. Molécule d'ADN de recombinaison selon la revendication 5, caractérisée en ce que le gène bactérien est le gène pour pénicillinase E. Coli.

Revendications pour l'Etat contractant suivant: AT

1. Procédé de production d'une protéine de fusion sélectionnée ou d'une polypeptide de fusion sélectionnée, protéine ou polypeptide caractérisée par une protéine ou une polypeptide non bactérienne fusionnée bout à bout à une partie d'une protéine porteuse bactérienne extra-cellulaire ou périplasmique, caractérisé en ce qu'on sépare le gène bactérien de la protéine porteuse extra-cellulaire ou périplasmique à l'intérieur de la partie du gène bactérien codant la protéine porteuse bactérienne, on insère dans le site de la séparation, par une étape recombinant un fragment d'ADN non bactérien qui code une protéine ou une polypeptide normalement éliminée à travers une membrane de la cellule dans laquelle elle est produite au naturel, on transforme un porteur bactérien par le gène recombiné et on cultive la bactérie transformée pour éliminer la protéine de fusion sélectionnée ou la polypeptide de fusion sélectionnée, à travers une membrane de la bactérie transformée.

2. Procédé selon la revendication 1, caractérisé en ce que la protéine porteuse est de la pénicillinase E. Coli.

3.Procédé selon l'une des revendications 1 et 2, caractérisé en ce que la proteine non bactérienne sélectionnée est la pro-insuline.

4. Procédé selon la revendication 1, caractérisé en ce que la protéine porteuse est la pénicillinase E. Coli et en ce que le gène bactérien est séparé au point de restriction Pst I.

5. Procédé selon la revendication 1, caractérisé en ce que le gène non bactérien a été introduit dans le gène bactérien au niveau du site de restriction d'endonucléase.

6. Procédé selon la revendication 5, caractérisé en ce que le gène bactérien est le gène de pénicillinase E. Coli.

7. Procédé selon la revendication 5, caractérisé en ce que le gène non bactérien est un gène de codage d'une protéine sélectionnée ou d'une polypeptide sélectionnée dans le groupe formé par la proinsuline, l'interféron, l'hormone de croissance humaine, des protéines de cellules eukaryotiques, des protéines virales et autres produits pharmacologiquement actifs.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten: BE, CH, DE, FR, GB, IT, LU, NL, SE

1. Verfahren zur Herstellung eines gewünschten Fusionsproteins oder Fusionspolypeptids, wobei das Protein oder Polypeptid dadurch gekennzeichnet ist, daß das Ende eines nicht-bakteriellen Proteins oder Polypeptids an das Ende eines Teils eines bakteriellen extrazellulären oder periplasmatischen Trägerproteins geknüpft ist, gekennzeichnet durch Schneiden des bakteriellen Gens für das extrazelluläre oder periplasmatische Trägerprotein innerhalb des Teils des bakteriellen Gens, das für das bakterielle Trägerprotein codiert, Einfügen eines nichtbakteriellen DNA-Fragments in die Schnittstelle durch einen Rekombinationsschritt, wobei das DNA-Fragment für ein Protein oder Polypeptid codiert, das normalerweise durch eine Membran der Zelle, in der es in der Natur gebildet wird, ausgeschleust wird, Transformieren eines bakteriellen Wirts mit dem rekombinierten Gen und Züchten der transformierten Bakterien, so daß das gewünschte Fusionsprotein oder Fusionspolypeptid durch die Membran des transformierten Bakteriums ausgeschleust wird.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß das Trägerprotein E.coli-Penicillinase ist.

3. Verfahren nach einem der Ansprüche 1 oder 2, dadurch gekennzeichnet, daß das gewünschte nicht-bakterielle Protein Proinsulin ist.

4. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß das Trägerprotein E.coli-Penicillinase ist und das bakterielle Gen an der PstI-Schnittstelle geschnitten wird.

5. Rekombinantes DNA-Molekül mit einem für ein extrazelluläres oder periplasmatisches Trägerprotein codierenden bakteriellen Gen und einem für ein gewünschtes Protein oder Polypeptid codierenden nicht-bakteriellen Gen, wobei das Protein oder Polypeptid normalerweise durch eine Membran der Zelle, in der es in der Natur gebildet wird, ausgeschleust wird, und wobei das Ende des nicht-bakteriellen Gens in das bakterielle Gen innerhalb des Teils des bakteriellen Gens eingefügt und mit dem Ende eines Teils davon verbunden würde, der für das bakterielle Trägerprotein codiert.

6. Rekombinantes DNA-Molekül nach Anspruch 5, wobei das nicht-bakterielle Gen in das bakterielle Gen an einer Restriktionsendonuclease-Schnittstelle eingefügt ist.

7. Rekombinantes DNA-Molekül nach Anspruch 6, wobei die Restriktionsendonuclease-Schnittstelle die PstI-Schnittstelle ist.

8. Rekombinantes DNA-Molekül nach Anspruch 5, wobei das bakterielle Gen das Gen für E.coli-Penicillinase ist.

Patentansprüche für folgende Vertragsstaat: AT

1. Verfahren zur Herstellung eines gewünschten Fusionsproteins oder Fusionspolypeptids, wobei das Protein oder Polypeptid dadurch gekennzeichnet ist, daß das Ende eines nicht-bakteriellen Proteins oder Polypeptids an das Ende eines Teils eines bakteriellen extrazellulären oder periplasmatischen Trägerproteins geknüpft ist, gekennzeichnet durch Schneiden des bakteriellen Gens für das extrazelluläre oder periplasmatische Trägerprotein innerhalb des Teils des bakteriellen Gens, das für das bakterielle Trägerprotein codiert, Einfügen eines nichtbakteriellen DNA-Fragments in die Schnittstelle durch einen Rekombinationsschritt, wobei das DNA-Fragment für ein Protein oder Polypeptid codiert, das normalerweise durch eine Membran der Zelle, in der es in der Natur gebildet wird, ausgeschleust wird, Transformieren eines bakteriellen Wirts mit dem rekombinierten Gen und Züchten der transformierten Bakterien, so daß das gewünschte Fusionsprotein oder Fusionspolypeptid durch die Membran des transformierten Bakteriums ausgeschleust wird.

2. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß das Trägerprotein E.coli-Penicillinase ist.

3. Verfahren nach einem der Ansprüche 1 oder 2, dadurch gekennzeichnet, daß das gewünschte nicht-bakterielle Protein Proinsulin ist.

4. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß das Trägerprotein E.coli-Penicillinase ist und das bakterielle Gen an der PstI-Schnittstelle geschnitten wird.

5. Verfahren nach Anspruch 1, dadurch gekennzeichnet, daß das nicht-bakterielle Gen in das bakterielle Gen an einer Restriktionsendonuclease-Schnittstelle eingeführt worden ist.

6. Verfahren nach Anspruch 5, dadurch gekennzeichnet, daß das bakterielle Gen das Gen für E.coli-Penicillinase ist.

7. Verfahren nach Anspruch 5, dadurch gekennzeichnet, daß das nicht-bakterielle Gen ein Gen ist, das für ein gewünschtes Protein oder Polypeptid aus der Gruppe Proinsulin, Interferon, menschliches Wachstumshormon, eukaryotische Zellproteine, virale Proteine und andere pharmakologisch wirksame Produkte codiert.

R1

GAATTCTTGAAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCAGGTGGCACTTTTCGGGGAAATGTG

20 40 60 80 100

CTTAAGAACTTCTGCTTTCCCGGAGCACTATGCGGATAAAAATATCCAATTACAGTACTATTATTACCAAAGAATCTGCAGTCCACCGTGAAAAGCCCCTTTACAC

CGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCCTGATAAATGCTTCAATAATATTGAAAAAGGAAG

120 140 160 180 200

GCGCCTTGGGGATAAACAAATAAAAAGATTTATGTAAGTTTATACATAGGCGAGTACTCTGTTATTGGGACTATTTACGAAGTTATTATAACTTTTTCCTTC

10 20 30

MetSerIleGlnHisPheArgValAlaLeuIleProPhePheAlaAlaPheCysLeuProValPheAlaHisProGluThrLeuValLysValLysAsp

AGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGAT

220 240 260 280 300

TCATACTCATAAGTTGTAAAGGCACAGCGGGAATAAGGGAAAAAACGCCGTAAAACGGAAGGACAAAAACGAGTGGGTCTTTGCGACCACTTTCATTTTCTA

40 50 60

AlaGluAspGlnLeuGlyAlaArgValGlyTyrIleGluLeuAspLeuAsnSerGlyLysIleLeuGluSerPheArgProGluGluArgPheProMetMet

GCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATG

320 340 360 380 400

CGACTTCTAGTCAACCCACGTGCTCACCCAATGTAGCTTGACCTAGAGTTGTCGCCATTCTAGGAACTCTCAAAAGCGGGGCTTCTTGCAAAAGGTTACTAC

70 80 90 100

SerThrPheLysValLeuLeuCysGlyAlaValLeuSerArgValAspAlaGlyGlnGluGlnLeuGlyArgArgIleHisTyrSerGlnAsnAspLeuVal

AGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTGTTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTT

420 440 460 480 500

TCGTGAAAATTTCAAGACGATACACCGCGCCATAATAGGGCACAACTGCGGCCCGTTCTCGTTGAGCCAGCGGCGTATGTGATAAGAGTCTTACTGAACCAA

FIG. 1

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110 120 130
GluTyrSerProValThrGluLysHisLeuThrAspGlyMetThrValArgGluLeuCysSerAlaAlaIleThrMetSerAspAsnThrAlaAlaAsnLeu
GAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTA
520 540 560 580 600
CTCATGAGTGGTCAGTGTCTTTTCGTAGAATGCCTACCGTACTGTCATTCTCTTAATACGTCACGACGGTATTGGTACTCACTATTGTGACGCCGGTTGAAT

140 150 160
LeuLeuThrThrIleGlyGlyProLysGluLeuThrAlaPheLeuHisAsnMetGlyAspHisValThrArgLeuAspArgTrpGluProGluLeuAsnGlu
CTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAA
620 640 660 680 700
GAAGACTGTTGCTAGCCTCCTGGCTTCCTCGATTGGCGAAAAAACGTGTTGTACCCCCTAGTACATTGAGCGGAACTAGCAACCCTTGGCCTCGACTTACTT

170 180 190 200
AlaIleProAsnAspGluArgAspThrThrMetProAlaAlaMetAlaThrThrLeuArgLysLeuLeuThrGlyGluLeuLeuThrLeuAlaSerArgGln
GCCATACCAAACGACGAGCGTGACACCACGATGCCTGCAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAA
720 740 760 780 800
CGGTATGGTTTGCTGCTCGCACTGTGGTGCTACGGACGTCGTTACCGTTGTTGCAACGCGTTTGATAATTGACCGCTTGATGAATGAGATCGAAGGGCCGTT

210 220 230
GlnLeuIleAspTrpMetGluAlaAspLysValAlaGlyProLeuLeuArgSerAlaLeuProAlaGlyTrpPheIleAlaAspLysSerGlyAlaGlyGlu
CAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAG
820 840 860 880 900
GTTAATTATCTGACCTACCTCCGCCTATTTCAACGTCCTGGTGAAGACGCGAGCCGGGAAGGCCGACCGACCAAATAACGACTATTTAGACCTCGGCCACTC

240 250 260 270
ArgGlySerArgGlyIleIleAlaAlaLeuGlyProAspGlyLysProSerArgIleValValIleTyrThrThrGlySerGlnAlaThrMetAspGluArg
CGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGA
920 940 960 980 1000 1020
GCACCCAGAGCGCCATAGTAACGTCGTGACCCCGGTCTACCATTCGGGAGGGCATAGCATCAATAGATGTGCTGCCCCTCAGTCCGTTGATACCTACTTGCT
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FIG. 2

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                    280                 286
AsnArgGlnIleAlaGluIleGlyAlaSerLeuIleLysHisTrp
AATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGA
               1040                1060                1080                1100
TTATCTGTCTAGCGACTCTATCCACGGAGTGACTAATTCGTAACCATTGACAGTCTGGTTCAAATGAGTATATATGAAATCT
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FIG. 3